

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification⁶ : C12N 1/14, A01N 63/00, 63/04</p>	<p>A1</p>	<p>(11) International Publication Number: WO 95/10597 (43) International Publication Date: 20 April 1995 (20.04.95)</p>
<p>(21) International Application Number: PCT/US94/11542 (22) International Filing Date: 12 October 1994 (12.10.94) (30) Priority Data: 08/134,563 12 October 1993 (12.10.93) US (71) Applicant: MYCOTECH CORPORATION [US/US]; 630 Utah Avenue, Butte, MT 59702 (US). (72) Inventors: BRADLEY, Clifford, A.; 1923 Argyle Street, Butte, MT 59701 (US). BRITTON, James, H.; 2324 Locust Street, Butte, MT 59701 (US). (74) Agents: HANLEY, Elizabeth, A. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).</p>		<p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).</p> <p>Published With international search report.</p>
<p>(54) Title: FORMULATIONS OF ENTOMOPATHOGENIC FUNGI FOR USE AS BIOLOGICAL INSECTICIDES</p> <p>(57) Abstract</p> <p>Entomopathogenic formulations that include conidia of an entomopathogenic fungus and a carrier are described. Carriers include oils, emulsions, and suspensions. Methods of killing insects such as grasshoppers using the aforementioned formulations are also described.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

FORMULATIONS OF ENTOMOPATHOGENIC FUNGI FOR USE AS BIOLOGICAL INSECTICIDES

5

RELATED APPLICATIONS

This application is related to applications entitled "A Solid Culture Substrate Including Barley" and "Mycoinsecticides Against an Insect of the Grasshopper Family", filed on even date herewith, the contents of each of the aforementioned applications are hereby expressly incorporated.

BACKGROUND OF THE INVENTION

Entomopathogenic fungi or insect pathogenic fungi have the potential to be a new class of bioinsecticides suitable for wide spread applications in agriculture. The mode of action of the fungi, penetration through the insect cuticle, makes fungi effective against a wide range of insect pests that cannot be controlled effectively by bacterial, viral, or protozoan pathogens that act through ingestion.

Insect pathogenicity occurs in a broad range of fungi including the genera *Beauveria*, *Metarhizium*, *Nomuraea*, *Verticilium*, *Aschersonia*, *Hirsutella*, and *Paecilomyces*. Common traits of all these fungi are that they all produce conidia, conidia are the infective form of the organism and all have been shown to produce conidia *in vitro*.

Researchers have attempted to determine formulations in which the conidia of the entomopathogenic fungus remain viable. Daoust *et al.* (*J. Invert. Path.* 41:151-160 (1983)) discuss the effect of formulation on the viability of *Metarhizium anisopliae* conidia. In their experiments, fourteen oils were tested including mineral oil, cod liver oil and twelve vegetable oils. Daoust *et al.* teach that the fourteen oils were highly detrimental to the viability of the conidia after two months

- 2 -

of storage. They also teach that organic acids and water were lethal to the conidia. In another paper, Prior et al. found that a conidial suspension of *Beauveria bassiana* in coconut oil, water and 0.01% Tween-80 was infective against the cocoa weevil pest, *Pantorhytes* 5 *plutus*. In addition, it is disclosed that the suspension can be stored for 40 days in the refrigerator.

Generally, vegetable oils have been used as carriers for entomopathogenic fungi. However, vegetable oils do not confer long 10 term shelf life and do not have good spray characteristics.

Conidia of *Beauveria bassiana* and other species of entomopathogenic fungi are strongly hydrophobic and difficult to suspend in water. Many applications of fungi use water, detergent 15 suspensions, or wettable powders that use selected clays as suspension aids or dry powder. However, in water suspensions, conidia can germinate and lose their infectivity after only twenty-four hours.

SUMMARY OF THE INVENTION

20 Commercial use of fungal insect pathogens as mycopesticides requires that fungal conidia remain viable and infective during and after storage and transport and that fungal conidia be effectively delivered to the target insect in a viable form. It also is important that fungal conidia be stored and shipped economically, and 25 applied effectively in a wide range of conditions with a variety of application equipment.

The present invention provides entomopathogenic formulations that include conidia of an entomopathogenic fungus 30 suspended in a carrier. Carriers include oils, emulsions, and suspensions. The fungus-oil suspension can be emulsified with water and can contain other additives. The fungus-oil suspension and formulations prepared from the fungus-oil suspension have the advantages of increased shelf life and temperature stability compared

- 3 -

with dry conidia preparations or water suspensions, increased protection of conidia from ultraviolet radiation and greater infectivity than conidia suspended in water. Also provided are methods of killing insects using the formulations of the present invention.

5

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B are graphs depicting mortality results of bioassays involving oil and clay/water formulations of *Beauveria bassiana* against nymphs of *Oedaleus senegalensis*.

10

Figure 2 is a bar graph which shows the grasshopper species composition in treated and untreated plots prior to application of *Beauveria bassiana*.

15

Figure 3 is a bar graph which shows mortality results of six grasshopper species collected from field test plots that have been treated with *Beauveria bassiana*.

20

Figure 4 is a bar graph which shows mortality results of grasshoppers treated aerially with BbGHA1991 and held on native rangeland in cages.

25

Figure 5 is a graph depicting changes in grasshopper population as determined from ring counts after application of *Beauveria bassiana*.

30

Figure 6 is a graph depicting reduction in grasshopper population density after a ground application test of BbGHA1991, an isolate of *Beauveria bassiana*, and controls.

Figure 7 is a graph depicting cumulative mortality of grasshoppers collected one hour post-treatment in a test where BbGHA1991 and the control were applied from the ground.

- 4 -

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an entomopathogenic formulation that includes conidia of an entomopathogenic fungus and a carrier oil. A small volume of the formulation is capable of being spread over a large geographical area or water emulsions can be prepared from the oil formulations for high volume applications.

The language "entomopathogenic formulation" is intended to include a mixture of conidia of an entomopathogenic fungus and a carrier. The carrier is a substance capable of dispensing the fungus appropriately without adversely affecting the fungus' ability to perform its intended function. Carriers of the present invention include oils, emulsions, and suspensions.

The term "conidia" is art-recognized and is intended to include asexual spores characteristic of fungi. Conidia of a fungus can be counted and used as units of measure of the fungus, for example, with respect to viability and LD₅₀.

The language "entomopathogenic fungus" means a fungus which is capable of killing an insect. Such a fungus is considered a mycopesticide. Entomopathogenic fungi include those strains or isolates of fungal species in the class *Hyphomycetes* which possess characteristics allowing them to be virulent against insects. These characteristics include formation of stable infective conidia. An effective entomopathogenic fungus preferably is lethal for target insects but less harmful for non-target insects. Also, the entomopathogenic fungus preferably does not harm vegetation or animals who might come in contact with it.

The term "non-phytotoxic" is intended to include a substance that is not significantly inhibitory to the growth of or poisonous to plants at application rates used in insect control or at conidia levels necessary to kill the target insect. A non-phytotoxic

- 5 -

substance such as a horticultural oil cannot adversely affect any vegetation with which the substance comes in contact. Non-phytotoxic substances include horticultural oils such as Sunspray® 6N and Sunspray® 6E and agricultural oils such as Sunspray® 7E and
5 Sunspray® 7N.

The term "oil" is art-recognized and is intended to include a substance which is an unctuous, viscous liquid at ordinary temperatures. Oils can be derived from either petroleum or from
10 vegetables. Oils include light paraffinic oils such as Sunspray® 6N, Sunspray® 6E, or Sunspray® 7E as well as other petroleum-based oils and vegetable oils such as those derived from corn, cottonseed, soy beans, palm or coconut, rape seed, and sunflower seed. In addition, conidia are killed by exposure to sunlight, particularly ultraviolet
15 wavelengths. Oils preferably include those that can protect entomopathogenic fungal conidia from harmful ultraviolet radiation. Formulations which protect conidia from sunlight damage are advantageous in increasing persistence of conidia in the field after spraying. Conidia also can be killed by exposure to elevated
20 temperatures. Oils included are those that do not adversely affect, or preferably those which enhance, conidia stability.

The language "petroleum based oil" includes oils that are derived from petroleum including light paraffinic oils. Petroleum based
25 oils typically have distillation midpoints in the range of 404-435°F. In addition, light paraffinic petroleum oils can have an unsulfonated residue of at least 90 and a CAS number 64741-89-5. Light paraffinic petroleum oils include Sunspray® 6N, Sunspray® 6E, and Sunspray® 7E. Petroleum based oils preferably include those which do not
30 adversely affect the viability and/or virulence of the conidia derived from the fungus. Petroleum based oil for purposes of this invention is not intended to include mineral oil which adversely affects the viability and/or virulence of the conidia derived from the fungus.

- 6 -

The language "fungus" is art-recognized. The fungi of the invention can be entomopathogenic or can produce commercially useful enzymes such as amylase and/or ligninase. Examples of fungus include fungi of the subdivision *Deuteromycotina* (or *Deuteromycetes*) and
5 fungi of the class *Hyphomycetes*. Generally, fungi of the class *Hyphomycetes* can produce conidia. Examples of entomopathogenic fungus genera include *Beauveria*, *Metarhizium*, *Paecilomyces*, *Tolypocladium*, *Aspergillus*, *Culicinonyces*, *Nomuraea*, *Sorospora*, *Verticillium*, and *Hirsutella*. Examples of fungus genera that are not
10 entomopathogenic include *Trichoderma* and *Alternaria*. Examples of species of fungus include *Beauveria bassiana*, *Metarhizium flavoviride*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus*, *Paecilomyces farinosus*, *Nomuraea rileyii*, *Aspergillus niger*, *Aspergillus awamori*, *Trichoderma viride*, and *Trichoderma harzianum*. Fungus includes
15 those strains or isolates of fungal species that possess characteristics allowing them to be virulent against insects. These characteristics include formation of stable, infective conidia.

The present invention also provides an entomopathogenic
20 formulation that includes conidia of an entomopathogenic fungus and an emulsion. The term "emulsion" is intended to include mixtures of two liquids not mutually soluble which are capable of suspending the conidia of an entomopathogenic fungus. Emulsions include mixtures of oil and water. In an oil and water emulsion, the oil can aid in
25 suspending the hydrophobic conidia and allow for high volume dispersion of the fungus. In water suspension, conidia of the entomopathogenic fungus typically germinate and are noninfective within twenty-four hours. However, the oil in the emulsion surrounds the conidia and can retard germination for several days, thus, allowing
30 storage of the formulation after addition of water for several days prior to application.

The present invention further provides an entomopathogenic formulation that includes conidia of an

- 7 -

entomopathogenic fungus and a non-phytotoxic suspension as well as an entomopathogenic formulation that includes conidia of an entomopathogenic fungus, a non-phytotoxic suspension and petroleum jelly. Also included are oil or oil water emulsions with spreaders, stickers, or other additives used for purposes such as aiding conidia suspension or dispersal or enhancing ultraviolet protection.

The term "suspension" is intended to include a solid substance which, in a particulate form, is mixed with a fluid but which remains undissolved. Suspensions include those where clay or diatomaceous earth is suspended in water or in oil/water emulsions. In clay/water suspensions or clay/water/oil emulsions, the clay helps the hydrophobic conidia to be suspended in water for greater dispersion and longer shelf life.

The term "clay" is art-recognized and is intended to include a natural earthy material that is plastic when wet and can act as a carrier in suspension of conidia of a fungus. In an emulsifiable suspension, the clay can stabilize the oil and water emulsion and allow the liquids to remain emulsified even in unagitated spray equipment. The amount of clay needed depends on the final water volume to be added and can be determined by routine experimentation. Examples of clays within the scope of this invention include attapulgite clay, kaolin, and bentonite.

The language "petroleum jelly" is art-recognized and is intended to include a gelatinous mass obtained from petroleum. Petroleum jelly includes a smooth, semisolid blend of mineral oil with waxes crystallized from the residual type of petroleum lubricating oil. Petroleum jelly can make the droplets of conidia/oil suspensions and emulsions more uniform and denser. Addition of petroleum jelly reduces spray drift in aerial application and provides additional protection of the fungal conidia from ultraviolet radiation. Petroleum

- 8 -

jelly also increases viscosity and aids in maintaining conidia in suspension, e.g., during storage.

Generally, conidia of an entomopathogenic fungus are hydrophobic. Oils, emulsions, and suspensions of clay in oil or clay in an oil/water emulsion can keep the conidia suspended in liquid form for greater ease of delivery. The advantages of these formulations are ease of delivery, temperature stability, and longer shelf life. When the conidia are suspended in an oil, only a very low volume of oil is needed. Conidia disperse uniformly in oil and disperse uniformly upon application in small volumes. Water suspensions could be applied at equally low volumes but the conidia would not be as uniformly dispersed. In addition, conidia are more stable under exposure to elevated temperatures when suspended in oils. Selected carrier oils include light paraffinic oils. Also, a suspension of conidia in water generally has to be used within a few days. However, a suspension of conidia in oil has a much longer shelf life, e.g., one of months instead of days.

The language "small volume" is intended to include volumes recognized by those of skill in the art as being small volumes for suspending entomopathogenic fungal conidia. Small volume includes the amount of liquid necessary or sufficient to suspend the conidia of the entomopathogenic fungus. Small volume also includes the minimum amount of a liquid such as a carrier oil needed to suspend the conidia. In addition, small volume includes that volume of formulation used in ultra low volume applications, e.g. aerial applications. Examples of small volume amounts and applications are described in the examples below.

30

The language "high volume" is intended to include volumes recognized by those of skill in the art as being high volumes for suspending entomopathogenic fungal conidia. High volume includes the amount of oil necessary to suspend the conidia to which

- 9 -

water has been added to form an emulsion. High volumes of conidia suspension can be used to apply the conidia to an affected area using ground equipment such as a tractor pulling a spray apparatus. Typically, ground equipment move across a field at a much slower rate
5 than an airplane and high volumes are needed to ensure that the correct amount of conidia per unit area is applied. Examples of high volume amounts and applications are described in the examples below.

The language "being capable of spreading" is intended to
10 include the ability to be applied over an area either evenly or unevenly. The language includes substances such as oils or emulsions that can be evenly or unevenly distributed over a geographical area by land or by air.

The language "large area" is intended to include an extent
15 of space or surface that would be recognized by those of skill in the art as being a large area. Large areas include parcels or portions of land on which insects are present.

The language "viscosity that allows the entomopathogenic
20 formulation to be sprayed through a nozzle" is intended to include liquids whose ability to resist a force that causes the liquid to flow is such that the liquid can be forced through a nozzle to form a spray. The viscosity cannot be so high that it clogs the nozzle when it is forced
25 through the opening.

The term "nozzle" is intended to include a spout or
terminal discharging pipe through which a substance flows. Nozzle
includes a spout that causes a liquid to become a spray. A nozzle can
30 be used on airplane spray booms or ground sprayers to finely divide the flow of entomopathogenic formulation into a spray for efficient application.

- 10 -

Next, the present invention provides methods of killing insects using the aforementioned formulations. The formulations can be administered to an affected geographical area by either land or air.

5 The language "an affected geographical area" is intended to include a parcel or portion of land which has insects present on that land. Affected geographical areas include those areas which contain large numbers of insects that cause damage to vegetation or crops.

10 The term "applying" is intended to include a method of bringing the conidia of an entomopathogenic fungus in contact, either externally or internally, with an insect, for example, by a droplet directly contacting an insect or the insect's body coming in contact with the conidia as the insect travels across a leaf. Application includes
15 direct application of the conidia of an entomopathogenic fungus to the insect as in topical application. Application also includes spraying an affected geographical area with the conidia in liquid form. The suspended conidia can be sprayed on the affected geographical area from the air such as from a low flying plane or from the ground such as
20 by an individual with a tank sprayer. Application also includes spraying vegetation or soil and subsequent contact between an insect and conidia on sprayed vegetation or soil.

 The term "insect" is art-recognized. Examples of insects
25 include grasshopper, locust, white fly, gypsy moth, Colorado potato beetle, corn borer, citrus root weevil, corn root worm, and thrip.

 The following invention is further illustrated by the following non-limiting examples. The contents of all cited copending
30 applications, issued patents, and published references are hereby expressly incorporated by reference.

- 11 -

EXAMPLES

Example 1: *Preparation of Beauveria bassiana Conidia*

5 Conidia preparations of *Beauveria bassiana* strain BbGHA1991 were produced in a series of solid cultures using 1.5 or 3 kg dry weight culture substrate as follows:

10 The strain was maintained as a dried laboratory solid culture containing viable conidia stored at 4°C. Broth cultures of composition described below were inoculated with conidia from this maintenance culture and incubated at 25°C on a rotary shaking water bath for three to six days. Broth culture medium results in production of high numbers of single-celled blastospores. Typical broth cultures
15 contain in excess of 1×10^8 blastospores per ml. For some larger cultures, 100ml of broth culture were transferred to 1.5 liters broth in 2800ml flasks incubated at 25°C with approximately 500cc/minute sparged air flow.

20 Inoculum Culture Medium

	Glucose	40g/l
	KNO ₃	10g/l
	KH ₂ PO ₄	5g/l
25	MgSO ₄	1g/l
	CaCl ₂	0.05g/l
	Yeast Extract	2g/l

30 Solid culture substrate was prepared by mixing equal parts by weight of dry barley flakes and the same inoculum culture medium described above except that glucose was omitted. Wetted barley was autoclaved in polypropylene bags for 20 minutes to one hour (depending on volume) at 15psi at 121°C, cooled, and inoculated by transferring broth cultures directly to bags of substrate which were
35 mixed externally by hand. Generally 1.5 kg dry weight barley flakes

- 12 -

were mixed with 1500ml nutrient solution and autoclaved in one bag, cooled and inoculated with 300ml of broth culture. Inoculated solid substrate was transferred to an autoclave-sterilized polycarbonate box 27cm x 48cm x 15cm fitted with a screen bottom and connectors for air inlet and outlet. The substrate formed a bed about eight to ten centimeters deep on the screen. In some cultures, 3kg dry weight flakes were processed and incubated in 18" diameter x 24" deep round steel vessels fitted with screens. The culture beds were about 20 to 30cm deep. Cultures were incubated at 20-30°C for 10 days with an air flow of about 0.5 to 2 liters/minute. Air flow varied to maintain culture temperature. After eight to twelve days incubation, cultures were transferred to a dryer consisting of screens and equipped with a fan. Cultures were spread on screens and dried to a final moisture content of less than 10% with a flow of dry air at 20-25°C.

15

Dried cultures were passed through a Wiley mill which had cutting blades removed. This removed conidia from dried barley flakes by turbulence and particle to particle abrasion without significantly reducing the size of residual barley flakes. The mill discharged to a covered vibrating mesh (US Standard) screen fitted with a cover. Material that passed through the screen was weighed and assayed for the concentration of viable conidia by methods described below:

25

0.1g conidia preparation is weighted into 9.9ml
0.1% Tween 80 solution in a Potter-Elvehjem homogenizer tube

30

Conidia suspension was homogenized for two minutes and diluted as appropriate (generally diluted to certain or estimated 1×10^6 to 1×10^7 conidia/ml in the final dilution.) Conidia concentration was determined by microscopic count at 400x magnification using a hemocytometer. (Neubauer-Levy or Petroff-Hauser Chamber or equivalent). Viability was determined by placing a drop of diluted conidia suspension on

- 13 -

Sabaroud's Dextrose Agar Yeast Extract (SDAY, Difco) plate. The drop was covered with a sterile microscope cover slip and plates were incubated 16-20 hours at 25°C. Plates were examined at 400x and germinated and ungerminated conidia were each counted. Conidia were considered germinated if swollen or if a hypha was emerging from the conidia. Conidia suspensions were sampled in duplicate. For each sample, a total of at least 100 conidia were counted in at least three microscope fields of view.

Fifty six individual cultures containing either 1.5 or 3 kg dry weight substrate were produced in seven sets over a 2½ month period. Conidia concentration, total weight of recovered dry conidia powder and concentration of conidia in final powder were determined for each culture and for a final pool of conidia powder made by blending conidia from individual cultures. Total substrate input was 91 kg, total weight of recovered conidia powder 6823 kg, total conidia recovered was 5.17×10^{14} and concentration of final pooled conidia powder was 7.6×10^{10} conidia per gram.

Example 2: Preparation of Concentrated Oil Formulation for Ultra Low Volume Application

Beauveria bassiana conidia powder prepared as described in Example 1 was suspended at the rate of 180g per liter of Sunspray® 7N Agricultural Oil (CAS #64741-89-5) obtained from Sun Oil Company, Philadelphia, PA. Sunspray® 7N oil has a distillation midpoint of 404-435°F and unsulfonated residue of 90. The final preparation contained 1.25×10^{13} conidia per liter.

- 14 -

**Example 3: *Preparation of Concentrated Oil Formulation
for Ultra Low Volume Application***

5 *Beauveria bassiana* conidia powder prepared according to
Example 1 was suspended at the rate of 75g per liter of Sunspray® 6N
Horticultural Oil (CAS #64741-89-85). Sunspray® 6N oil has a
distillation midpoint of 404-424°F and unsulfonated residue of 90. The
final preparation contained 5×10^{12} conidia per liter.

10 **Example 4: *Preparation of Emulsifiable Oil Concentrate
Formulations***

15 *Beauveria bassiana* conidia powders were suspended in
Sunspray® oils as in Examples 2 and 3, except that the emulsifiable
oils designated Sunspray® 7E and Sunspray® 6E oils were used.

**Example 5: *Comparison of Concentrated Oil Formulations in Spray
Tower Tests on Grasshoppers***

20 Formulations in Sunspray® 7N and 6E oils were prepared
as described in Examples 2 and 4. Conidia powders for each
formulation were from the same production lot of the *Beauveria*
bassiana strain BbGHA1991. Final conidia concentrations of each
formulation was 2.6×10^9 per ml.

25 Effectiveness of each formulation in infecting
grasshoppers was evaluated in tests using a spray tower system at the
USDA APHIS Methods Development Center, Phoenix, AZ.

30 Formulations were sprayed by injecting a measured
volume of formulation into the air stream of an air brush set at a height
of 1.8m above a 14" diameter target area. Volume injected was 0.09 ml
and was calculated to be equivalent to an application rate of 1×10^{13}
conidia in 3,785 ml (one gallon) per acre.

- 15 -

Each formulation was sprayed on 3rd and 4th instar *Melanoplus sanguinipes*. For each formulation, ten separate, replicated sets of five grasshoppers (50 grasshoppers per formulation) were sprayed. An untreated control group consisting of 50 grasshoppers was handled as the test grasshoppers except that they were not sprayed. A "treated control" group consisting of 50 grasshoppers was sprayed with formulation oil without conidia. Grasshoppers were held individually in screen cap, four ounce cups and fed daily with a diet of romaine lettuce and oat cereal. Holding room conditions were 26-33°C, 25-42% rh, 14 hour light, 10 hour dark photo period. Table 1 shows mortality over 12 days in the treated and control groups.

Table 1

15

Spray Tower Tests Oil Formulations
Mean % Mortality, Days Post Treatment

	<u>02</u>	<u>04</u>	<u>06</u>	<u>08</u>	<u>10</u>	<u>12</u>	<u>14</u>
20 6E <i>B. bassiana</i>	02	08	36	50	74	78	82
7N <i>B. bassiana</i>	00	08	42	64	74	78	82
25 6E Oil only	00	10	12	13	13	13	13
7N Oil only	20	10	18	18	22	22	22
Untreated	00	08	08	10	15	15	15

30

- 16 -

Example 6: *Storage Stability BbGHA1991 in Oil Formulation*

Conidia powder produced in a series of bench scale cultures by the method of Example 1 were pooled. Conidia in powder
5 were 99% viable. Conidia powder was mixed in 20 gallons of Sunspray® 6E which was loaded into a spray plane owned and operated by the U.S. Department of Agriculture. On the first spray date, the plane circled test plots for about 30 minutes. Wind was too high to spray and the plane returned to the airport. Upon return to the airport, temperature
10 of the oil suspension was about 105°F, measured with a medical thermometer. The suspension was drained from the plane to pails which were stored overnight at about 4°C. The next morning the suspension was reloaded in the plane and a portion sprayed on a test plot. Unused suspension was drained into pails. Pails were stored at
15 cool room temperature for eleven months. At this time, samples were taken and conidia viability assayed. Viability after use in field trial with exposure to elevated temperature and approximately eleven months of storage was greater than 90%.

20 Example 7: *Storage Stability of Beauveria bassiana at 40°C in Sunspray® Horticultural Oils 6N and 6E*

Beauveria bassiana strain BbGHA1991 conidia powder prepared by the method of Example 1 was suspended in Sunspray® 6E
25 and 6N oils. Final concentration was 3.5×10^9 conidia per ml. Conidia suspensions in screw cap tubes were placed in an incubator set at 40°C. Unformulated dry conidia powder was also placed in a screw cap tube in the same 40°C incubator. At selected time intervals, tubes were sampled and assayed for conidia viability according to the method
30 described in Example 1. Results are shown in Table 2. Conidia formulated in Sunspray® 6N and 6E oils maintained 80% viability through 18 days of storage at 40°C.

- 17 -

Table 2

Conidia Viability with Storage in Sunspray® 6 Oil at 40°C

5	<u>% Conidia Viability</u>			
	Days at 40°C	Conidia Powder	Sunspray® 6E	Sunspray® 6N
	0	99	99	99
	5	95	95	95
10	11	76	90	91
	18	59	80	80
	27	11	10	10
	39	2	2	2

15

Example 8: *Storage Stability of Beauveria bassiana in Sunspray® Agricultural Oil 7E at Different Temperatures*

Conidia powder of three strains of *Beauveria bassiana* were produced according to the method described in Example 1. Strains were BbGHA1991, RS 252, originally isolated from Colorado Potato Beetle used commonly in Beauveria research, and a Mycotech strain, designated GMB6 isolated from Gypsy moth. Strain RS 252 was obtained from the USDA ARS collection of entomopathogenic fungi, Ithaca, New York.

Conidia powder from each strain was suspended at 0.5g per 10ml Sunspray® 7E oil in a series of separate screw cap vials. Samples of dry conidia powder were also placed in vials. Vials were placed in dark incubators at 25°, 32°, and 42°C. Vials were sampled at time intervals and conidia viability was determined by the method described in Example 1. Results are shown in Table 3.

- 18 -

Table 3

**Conidia Viability of Three *Beauveria bassiana* Strains
With Storage in Sunspray® 7E Oil**

		% Conidia Viability					
		<u>Dry Powder</u>			<u>Oil 7E Formulation</u>		
		25°C	32°C	42°C	25°C	32°C	42°C
5							
		<u>Days of Storage</u>					
		Strain GHA 1991					
10	0	99	99	99	99	99	99
	3	99	99	99	99	99	99
	6	99	99	99	99	99	99
	13	99	99	95	99	99	99
	20	99	99	90	99	99	80
15	27	95	95	51	95	95	50
		Strain RS252					
20	0	99	99	99	99	99	99
	3	99	99	99	99	99	99
	6	99	99	95	99	95	99
	13	99	99	80	99	99	80
	20	87	85	61	80	90	40
	27	80	25	30	90	85	41
25		Strain GMB6					
30	0	99	99	99	99	99	99
	3	99	99	95	99	99	99
	6	95	99	95	99	99	99
	13	99	95	90	99	99	90
	20	82	85	85	95	99	85
	27	95	85	--	95	95	75

- 19 -

Example 9: *Preparation of Emulsifiable Suspension (ES)*
Formulation

Conidia powder prepared according to Example 1, was
 5 used to prepare an ES. The purpose of the ES was to minimize the
 amount of oil and allow high volume application using water as a
 diluent. The ES was mixed in the following ratios:

An amount of conidia powder containing 1×10^{13} conidia
 10 was suspended in 400ml of Sunspray® 6E to form the ES. Prior to
 application, 320g attapulgite clay (Attaclay RVM, Englehard Company,
 Iselin, NJ) and water to a final volume of two liters was added and
 mixed. This mixture formed a stable emulsion in which the conidia are
 15 contained in oil droplets in a water emulsion. Attaclay served to
 stabilize the emulsion. Varying amounts of oil, water, and clay can be
 added to achieve a desired final volume. A range of final volume ratios
 of clay, oil, and water can be mixed to form emulsified concentrates as
 shown below. These ratios illustrate the basic concept of the ES. A
 wide range of final volume with variations in clay and water addition
 20 are possible.

One gallon per acre rate	Conidia Powder to equal 1×10^{13} (120-150g)
9.3 l/ha	350g clay
25 or	1000ml oil
1 gal./ha	water to 3,785 ml (one gallon)
20 l/ha rate	Conidia Powder to equal 2.5×10^{13} Conidia
	460g clay
30	5 liters oil
	water to 20 liters

- 20 -

Example 10: *Stability of Conidia in an Emulsifiable Suspension (ES) Formulation*

Conidia mixed in water typically germinate within 24
5 hours. As a result, water suspensions must be used immediately after
mixing. In the ES formulation described in Example 9, conidia do not
germinate for several days after water addition. This allows use of
formulation diluted to final volume over several days.

10 Conidia powder was formulated as an ES as described in
Example 9 with 1.5g conidia powder in 10ml Sunspray® 6E oil, 3.5g
Attaclay RVM and 25ml water. The mixture was stored at room
temperature and samples were observed daily at 400x in a phase
contrast microscope. After 10 days, conidia had not germinated. At
15 seven and ten days, incubator samples were taken for viability assay.
Conidia viability was 80-90% in both samples.

Example 11: *Preparation of an ES Formulation with a Petroleum Jelly Additive*

20 Emulsifiable concentrate was prepared as in Example 9 in
the following ratio (equivalent to 1×10^{13} conidia in two liters final
volume per acre): 0.6g conidia powder, 4ml Sunspray® 6E oil, 3.2g
Attaclay to a final volume of 10 ml with water. Prior to mixing, 5%
25 (w/w) petroleum jelly was added to the oil/water/clay suspension.

The ES containing petroleum jelly was sprayed from the
spray tower described in Example 5 onto microscope slides. Droplets
of ES with and without petroleum jelly were examined by microscope.
30 ES with petroleum jelly formed more uniform droplets with conidia and
clay particles concentrated in the center. Droplets were also more
raised. These characteristics can be expected to provide a denser
droplet that will reduce spray drift in aerial application and provide
additional protection from ultraviolet light.

- 21 -

Example 12: Spray Tower Test of ES Formulations

Duplicate ES formulations were prepared as described in Example 9 with 1.5g conidia powder in 10ml Sunspray® 6E oil, 5.25g Attaclay RVM and 27ml water. One sample was made the day prior to spray and one immediately prior to spray. Formulations were sprayed on 3rd instar *Melanoplus sanguinipes* using the spray tower and methods described in Example 5 except that 0.05ml of formulation was sprayed on each replicate of five grasshoppers. Mortality results are shown in Table 4 below. Results demonstrate the stability and infectivity of conidia in oil/clay/water emulsion suspension 24 hours after water addition.

Table 4**Spray Tower Test ES Formulations**

		% Mortality Days Post Treatment						
		02	04	06	08	10	12	14
	ES - Fresh	2	4	26	44	50	54	62
	ES - 24hr	2	4	37	50	60	69	73
	ES without conidia	0	2	6	12	21	21	21
	Untreated	2	2	4	8	11	11	13

Example 13: Comparison of oil and water formulation in bioassay

Beauveria bassiana BbGHA1991 conidia powder was suspended in Sunspray® 6E oil as per Example 4 and serially diluted with oil to achieve concentration ranges described below. Conidia powder from the same production batch was mixed with attapulgate clay at a ratio of 1.5 parts conidia powder to 3.5 parts clay, suspended in

- 22 -

water and serially diluted to the same range of final concentrations as the oil suspensions.

Oil and water suspensions were compared in a topical
5 bioassay on 3rd instar Senegalese grasshoppers, *Oedalium senegalensis*.
In this test, grasshoppers were treated individually with a single 0.25
microliter droplet delivered by microsyringe to the pronotum of the
insect. For each dose rate of each formulation, 30 grasshoppers were
treated, held individually in small plastic cups, fed daily with fresh,
10 native grass and monitored for mortality.

Doses were 1000, 10,000, 50,000 and 100,000 conidia in
0.25 microliter per insect. Mortality results are shown in Figures 1A
and 1B. With the oil suspension, both the rate of kill and final total kill
15 are greater than with the water suspension. The higher mortality rate is
particularly significant showing 90% mortality at six days with the oil
formulation compared with 70% using the water suspension.

Example 14: Sunlight Protection of Conidia with Oil Formulations

20

Protection provided by oil formulation was evaluated in
sunlight exposure tests conducted at DSET Laboratories, Phoenix,
Arizona.

25

Water suspension, oil and ES formulations containing
different additives were prepared as outlined in Table 6. All
formulations contained 0.3g conidia powder in 10ml final formulation
volume, equivalent to 1×10^{13} conidia in one gallon (3.785 liters).
Each formulation was applied from the spray tower described in
30 Example 5 onto 2x3cm microscope slide cover slips. Cover slips were
mounted at the edges in holders, with three cover slips per holder.
Replicated sets of holders were sprayed with each formulation.
Controls were sprayed cover slips held in the dark at room temperature
(20 to 25°C). Immediately after spraying, holders were transported to

- 23 -

DSET labs in a dark container. Transport and mounting time was approximately one hour. Holders were placed on a solar exposure test stand at DSET labs. Exposure testing is performed in accordance with ASTM G7-89, Section 4.2.5 utilizing the DSET Laboratories

5 "Trac-Rac" machine. The Trac-Rac is an equatorial mounted, single axis, follow-the-sun tracking device, used to increase the amount of radiation a specimen would receive in specified time period. The total amount of energy available to the test specimen is increased by maintaining near-normal incidence (noontime conditions) from sunrise
10 to sunset. The specimens were mounted unbacked with the sprayed surface toward the sun.

Temperatures were monitored every 30 seconds with a five minute average and maximum recorded every hour, 24 hours per
15 day utilizing two 30 AWG Type T thermocouples. One thermocouple was attached with thermally conductive epoxy to the back surface of an unsprayed cover slip. The other thermocouple was located in a shaded, well-ventilated area to monitor the ambient air temperature. Temperature data was recorded with a Campbell Scientific Model 21x
20 datalogger.

At selected time intervals, one holder for each formulation was removed from the test stand. The three cover slips were removed from each holder and pressed formulation side down onto the surface of
25 Sabaroud's dextrose agar in petri dishes. This provided triplicate samples for each formulation at each time point. Petri dishes were incubated overnight at 25°C. Conidia viability was determined by microscope observation of conidia germination after overnight incubation. Conidia germination can be readily observed at 400x
30 magnification with phase contrast. The percentage of viable conidia was determined by counting the ratio of germinated to ungerminated conidia. A minimum of 100 total conidia were counted on each coverslip.

- 24 -

Solar radiation accumulation during the experiment is shown in Table 5. Table 6 shows average conidia viability for each formulation immediately prior to sunlight exposure, with 6.5 and 30 hour exposure and formulations held in the dark. Maximum
 5 temperature on the cover slips during the first 6.5 hours was 18.2°C and was 25.3°C during the second day of the test. Ambient temperature was within $\pm 2^\circ\text{C}$ of temperature on the cover slips. Minimum temperature was 8.1°C.

10 Conidia sprayed in water suspension showed 13% viability after 6.5 hours of exposure. All of the oil based formulations showed 78 to 94% viability after 6.5 hours. One formulation, #10, retained 10-25% viability with 30 hours exposure. Dark controls all
 15 oils in the carrier provided a significant degree of solar protection.

Table 5

20 Cumulative Solar Radiant Exposure
 During Conidia Viability Test

Mega Joule/m²

25	ULTRAVIOLET Radiant Exposure	Total
	Radiant Exposure	
	Day 1 (T=6.5hr)	0.74 29.1
30	Day 2 (T=30hr)	0.63 21.2
	Total (Cumulative)	1.37 50.3

- 25 -

Table 6

**Viability of *Beauveria bassiana* Conidia
Exposed to Sunlight**

5		<u>Formulation</u>	<u>T= 0</u>	<u>T=6.5hr</u>	<u>T=30hr</u>	<u>T=54hr</u>	<u>Dark 54 hr</u>
		1	95	13	0	0	68
		2	100	78	0	0	97
		3	97	73	0	0	96
10		4	97	83	0	0	94
		5	99	86	0	0	95
		6	95	94	0	0	95
		7	98	93	0	0	96
		8	98	94	<1	0	81
15		9	96	90	0	0	93
		10	92	83	10-25	0	93

		<u>Formulation</u>	
20	1	Spores suspended by homogenization in 0.1% Tween 80 in water	
	2	Sunspray® 7N Oil	
	3	Sunspray® 6E Oil	
	4	ES (0.3g spores, 1.6g clay*, 2ml 6E oil, 8ml H ₂ O)	
	5	Oil/Clay (2g clay in 10ml 7N)	
25	6	7N oil & 10% Petroleum Jelly (Protopet 15)	
	7	Oil/Petroleum Jelly/Clay (2g clay in 10ml 7N/10% petroleum jelly)	
	8	ES & Extra Clay (0.3g spores/2g clay/2ml 6E oil/8ml H ₂ O)	
30	9	ES & Petroleum Jelly (0.3g spores/1.6g clay/2ml 6E oil/10% petroleum jelly/8ml H ₂ O)	
		10 ES & Extra Clay & Petroleum Jelly (0.3g spores/2g clay/2ml 6E oil/10% petroleum jelly/8ml H ₂ O)	

*Attaclay RVM

- 26 -

Example 15: Aerial Application Field Trial of *Beauveria bassiana*
Ultra Low Volume Oil Formulation for Grasshopper
Control

5 Conidia preparations of *Beauveria bassiana* GHA1991
were produced in a series of bench scale cultures according to the
method described in Example 1. Conidia were produced and stored at
room temperature for 11-13 months. A portion of the conidia were
10 stored as dry powder and a portion was stored in Sunspray® 6E oil.
Formulations were prepared as described in Example 4 in Sunspray®
6E oil to a final concentration of 4.5×10^{12} viable conidia per liter.
Formulations were transported from Butte, Montana, under ambient
conditions to Bowman, North Dakota, and stored at ambient conditions.

15 Application was by air using an unmodified USDA
Cessna "Ag Truck", equipped with centrifugal pump and standard flat
fan spray nozzles (Tee-jet seize 8002). Application volume was
determined by pump pressure and the number of nozzles on the spray
20 boom. Volume was calibrated by measuring delivery volume of each
nozzle tip over 30 seconds in three replicated tests at the selected pump
pressure. Aircraft spray time over the plot was recorded by electronic
timer. Final application volume was confirmed by the difference in
reservoir volume before and after application. Application volume was
25 4.6 liters/hectare (2 quarts per acre). Conidia were applied at the rate of
 2×10^{13} conidia per hectare (8×10^{12} /acre). Application was in the early
morning with plots sprayed from a height of 10-20 feet. Application
coverage was monitored by inspection of oil sensitive spray cards
placed throughout the plots.

30 Field plots were mixed grass rangeland, typical of
improved grazing land in the Western United States. Grasshopper
species composition and population age structure were determined from
sweep net samples collected two days prior to application. Plots

- 27 -

contained a mixed population of grasshopper species and ages. The six most abundant species were monitored in efficacy evaluations. These species accounted for about 80% of the total population. Figure 2 lists grasshopper species composition in treated and untreated plots prior to application.

Application was to a single, four-hectare (ten acre) plot. Untreated and oil-only control plots were laid out in adjacent areas with very similar grass mix and grasshopper populations.

Efficacy was evaluated by three methods: observation of post-treatment grasshopper population samples, grasshoppers caged in the fields, and population density estimates.

For population samples, 50 grasshoppers of each species were collected by sweep net from test, untreated control and oil-only treated control plots on the day of application. Grasshoppers were held individually in 120cc plastic cups with screen lids, fed fresh, untreated field vegetation supplemented with romaine lettuce and oat cereal and monitored daily for mortality. Cups were held at ambient temperature for 12 days. Mycosis was confirmed by conidiation of *Beauveria bassiana* on grasshopper cadavers.

For field cages, 50 grasshoppers of each species were collected from test and from control plots and placed in ten liter cages constructed from plastic buckets and screen. Ten replicate cages with five grasshoppers per cage were used for each species. Field population densities were estimated by counts of grasshoppers in 0.1m² rings. Nine sites with ten rings per site were established.

Pre-spray grasshopper populations were estimated in counts on the two days immediately prior to application and post-spray counts made at three day intervals.

- 28 -

Both population samples (Figure 3) and field cages (Figure 4) showed high levels of mortality in treated plots compared with controls for all species in both trials. *Beauveria bassiana* mycosis was confirmed in more than 90% of dead grasshoppers in population samples from treated plots with no evidence of mycosis in dead grasshoppers from control plots.

Time to mortality varied between species with significant mortality beginning in as little as three days for some species and as much as five to six days for other species. These differences could be due to varying susceptibility of different grasshopper species, grasshopper age, or behavioral differences that result in varying exposure to conidia.

Comparisons of post-treatment population estimates in single, small test, and control plots are difficult to interpret. Figure 5 shows ring count density estimate after treatment. Significant population increases occurred in control plots with evidence of new hatch in later population samples. Weather was probably a factor in these trials. Through the first three days post-application, weather was clear with peak afternoon air temperatures of 27-39°C. Between the third and fourth days, a cold front brought rains and cooler temperature through day eight. During this period, peak afternoon temperatures were 9-15°C with minimums of 4.5-7°C, with intermittently heavy rain. The more rapid mortality of population samples held indoors, compared with those grasshoppers in outdoor field cages indicate infection was retarded at cooler temperatures.

Results obtained in this trial clearly demonstrate very high infection rates in grasshopper populations treated with ultra low volume aerial application of oil-formulated *Beauveria bassiana* conidia.

- 29 -

Example 16 *Comparison of ULV Oil and Emulsifiable Suspension Formulations in Field Trials*

Conidia powder was prepared as described in Example 1
5 and transported under ambient conditions. Powder contained 7×10^{10}
conidia per gram.

For ULV application, conidia powder was suspended in
Sunspray® 7N oil to a final concentration of 5×10^2 viable conidia per
10 liter (75g powder per liter) for application at five liters/hectare.
Emulsifiable suspension contained 300g conidia powder in five liters
Sunspray® 6E oil. The oil suspension was mixed with 460g Attaclay
RVM and water to a final volume of 20 liters for a one hectare
application.

15

The field test was conducted in cooperation with the
Instituto Nacional de Investigacao e Desenvolvimento Agraria (INIDA).
INIDA is located at Sao Jorge, Sao Tiago Island, Republic of Cabo
Verde. INIDA personnel chose a site three kilometers east of the town
20 of Tarrafal, on the north end of Sao Tiago island. The area was planted
with rows of acacia trees, the rows being three to six meters apart. The
ground was very rocky with sparse vegetation. The grasshopper
population was composed almost entirely of fifth instar larvae and adult
Oedaleus senegalensis.

25

A total of nine treatment plots, 100 x 200 meters (two
hectares) each, were laid out with a compass and flagging tape. Three
plots were treated with oil-formulated spores, three with emulsifiable
suspension-formulated spores, and three were left untreated.

30

The ULV oil formulation was sprayed at a rate of 2.5×10^{13} spores per hectare, in a volume of five liters per hectare, from
hand-held Microulva sprayers (Micron Sprayers, Ltd.).

- 30 -

The emulsifiable suspension-treated plots also received 2.5×10^{13} spores per hectare, but in a volume of 20 liters per hectare. Application was made via gasoline-powered backpack sprayers.

5 Approximately one hour after treatment, 250 grasshoppers were collected from each plot in sweep nets. These were transported back to the INIDA complex, separated into five cages of 50 grasshoppers each, and maintained in a laboratory. With three plots per treatment, this yielded a total sample of 750 grasshoppers per treatment,
10 or 2250 grasshoppers for the three treatments (oil, emulsifiable suspension, and untreated). Every day, INIDA personnel placed fresh grass in the cages and checked cages for mortality.

15 Daily grasshopper population density counts were made in the plots by a team of Cape Verde Plant Protection Service technicians using the PRIFAS "visual square meter" method. Each technician walked across a plot, pausing periodically to visually estimate the number of grasshoppers in a square meter. 100 square meters were counted per plot per day, and from this the average number
20 of grasshoppers per hectare was calculated.

25 The reduction in grasshopper population density is shown in Figure 6. After one week, populations treated with oil-formulated spores were reduced to 57% of untreated controls. ES-treated populations shrank to 54% of untreated. The seven day density reduction is statistically significant for both the oil and ES formulations ($p = 0.03$ and $p = 0.0204$, respectively).

30 Cumulative mortality of grasshoppers collected one hour post-treatment is shown in Figure 7. After seven days, greater than 98% of treated grasshoppers (both oil and ES treatments) were dead, compared with just 12% of untreated controls. Most insects died four to five days after treatment.

- 31 -

Example 18 *Preparation of Concentrated Oil Formulation
of Metarhizium flavoviride conidia*

Conidia of *Metarhizium flavoviride* (USDA ARSEF
5 2023) were produced by the method of Example 1, except that a slant
culture of the *Metarhizium flavoviride* strain was used as the source
culture. Final dried conidia powder contained 8.3×10^{10} viable conidia
per gram.

10 Conidia powder was suspended in Sunspray® 6E oil to a
final concentration of 5×10^{12} /liter. The formulation was transported
under ambient conditions, and used in a laboratory bioassay. The
formulation was assayed for conidia viability and used in a bioassay test
against grasshoppers. Viability was unchanged at 95%.

15

Example 19 *Oil Formulation of Beauveria bassiana Gypsy
Moth Strain*

A series of isolations were made from a *Beauveria*
20 *bassiana* infected Gypsy moth pupae collected from a woodlot in
Delaware. A conidia preparation was made directly from the infected
pupae and labeled Del B. Two single colony isolates made by dilution
of this preparation designated GMP1Y4 and GMP1W1 dry conidia
powder were prepared by the method described in Example 1. Conidia
25 were suspended in Sunspray® 6E oil at the rate of 1×10^8 conidia per
ml.

Conidia suspensions were sprayed on three foot lengths of
oak tree limbs at a rate of one ml per ten square inches of surface to a
30 final concentration of approximately 1×10^7 conidia per square inch of
surface. Limbs were allowed to dry for one to two hours after spraying
and were placed horizontally in trays. Gypsy moth larvae were released
on one end of the limb and a light bulb was placed at the other end of
the limb to attract the larvae. After the larvae had traversed the length

- 32 -

of the limb or had been exposed on the limb for one to three hours, the larvae were removed and held in cups on a standard gypsy moth diet and observed daily for mortality. Results are shown in Table 7. Strain GMP1W1 formulated in oil showed significant mortality.

5

Table 7

Laboratory Tests, Barrier Treatment *Beauveria bassiana* Delaware
Isolates in Conidia Oil Suspension

10

	<u>Isolate</u>	<u>Exposure Time</u>	<u>Mortality Observation</u>	
		<u>Hours</u>	<u>Days After Exposure</u>	<u>% Mortality</u>
15	Del B mix	0.75	11	50
			36	53
		0.75	11	70
			28	97
		3.5	14	89
20	Del B GMP 1Y4	1.25	10	88
		1.5	11	57
			21	63
25	Del B GMP 1W1	0.75	23	63
		1.0	15	100
		1.0	15	100
		1.75	9	50
30			26	93

- 33 -

Example 20 *Field Test of Oil Formulated Beauveria bassiana for Control of Gypsy Moths*

5 A strain of *Beauveria bassiana* ARSEF 201 which is virulent to gypsy moth in laboratory bioassay was used to prepare a dry conidia powder by the method of Example 1. Conidia powder was formulated in Sunspray®.6E oil to a final concentration of 4.8×10^8 /ml. The concentrated oil suspension was diluted 20:1 with water and shaken to form an emulsion at the time of application.

10

Field test plots were located in Delaware and composed of mixed hardwood woodlots, primarily white and red oak, sweet gum and beech. Two plots, one of three acres and one of 0.5 acre, isolated by open areas were used as test plots. Two control plots of similar size
15 were delineated.

Conidia emulsion suspensions were applied to the point of runoff with a backpack sprayer to the lower six to eight feet of tree trunk of all dominant trees in the test plots. The sprayed portion of
20 trees contained high numbers of larvae. Application was made when larvae were four to six instar and pupation was first observed. About 15 minutes after spraying, 100 larvae were removed from each test plot and held in a laboratory in standard diet cups for two weeks. Table 8 shows mortality in larvae in this contact spray test.

25

Table 8

Field Contact Spray Test

Beauveria bassiana Oil Emulsion Formulation on Gypsy Moth

30

% Mortality

Treatment	82.5
Control	17.5

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous
5 equivalents to the specific embodiments described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

- 35 -

CLAIMS

1. An entomopathogenic formulation comprising conidia of an entomopathogenic fungus and a petroleum based oil selected such that viability or virulence of the conidia are not adversely affected by the oil.
2. The entomopathogenic formulation of claim 1 wherein the petroleum based oil is a light paraffinic oil.
3. The entomopathogenic formulation of claim 2 wherein the light paraffinic oil is of the class of oils defined by CAS number 64741-89-5.
4. The entomopathogenic formulation of claim 1 wherein the oil does not adversely affect the viability of the conidia.
5. The entomopathogenic formulation of claim 1 wherein the entomopathogenic fungus comprises a fungus of the class *Hyphomycetes*.
6. The entomopathogenic formulation of claim 5 wherein the fungus of the class *Hyphomycetes* is selected from the group of genera consisting of *Beauveria*, *Metarhizium*, *Paecilomyces*, *Culicinomyces*, *Nomuraea*, *Sorospora*, *Tolypocladium*, *Verticillium*, and *Hirsutella*.
7. The entomopathogenic formulation of claim 6 wherein the entomopathogenic fungus is selected from the group consisting of *Beauveria bassiana*, *Metarhizium flavoviride*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus*, and *Paecilomyces farinosus*.

- 36 -

8. The entomopathogenic formulation of claim 2 wherein the light paraffinic petroleum oil is Sunspray® 7N Agricultural oil or Sunspray® 6N Horticultural oil.
- 5 9. The entomopathogenic formulation of claim 1 further characterized by a small volume of the formulation being capable of spreading over a large area.
- 10 10. The entomopathogenic formulation of claim 1 further characterized by a high volume of the formulation being capable of spreading over a large area.
- 15 11. The entomopathogenic formulation of claim 1 wherein the entomopathogenic formulation has a viscosity that allows the entomopathogenic formulation to be sprayed through a nozzle.
- 20 12. The entomopathogenic formulation of claim 1 wherein the conidia in the formulation retains its entomopathogenic activity for prolonged periods of time.
- 25 13. An entomopathogenic formulation comprising conidia of an entomopathogenic fungus and an emulsifiable carrier oil.
14. The entomopathogenic formulation of claim 1 wherein the entomopathogenic fungus comprises a fungus of the class *Hyphomycetes*.
- 30 15. The entomopathogenic formulation of claim 13 wherein the fungus of the class *Hyphomycetes* is selected from the group of genera consisting of *Beauveria*, *Metarhizium*, *Paecilomyces*, *Culicinomyces*, *Nomuraea*, *Sorospora*, *Tolypocladium*, *Verticillium*, and *Hirsutella*.

- 37 -

16. The entomopathogenic formulation of claim 15 wherein the entomopathogenic fungus is selected from the group consisting of *Beauveria bassiana*, *Metarhizium flavoviride*, *Metarhizium anisopliae*, *Paecilomyces fumusoroseus*, and *Paecilomyces farinosus*.

5

17. The entomopathogenic formulation of claim 13 further characterized by a small volume of the formulation being capable of spreading over a large area.

10

18. The entomopathogenic formulation of claim 13 further characterized by a high volume of the formulation being capable of spreading over a large area.

15

19. The entomopathogenic formulation of claim 13 wherein the entomopathogenic formulation has a viscosity that allows the entomopathogenic formulation to be sprayed through a nozzle.

20

20. The entomopathogenic formulation of claim 13 wherein the conidia in the formulation retains its entomopathogenic activity for prolonged periods of time.

21. An entomopathogenic formulation comprising conidia of an entomopathogenic fungus in an emulsion.

25

22. The entomopathogenic formulation of claim 21 wherein the emulsion is non-phytotoxic at levels used to kill target insects.

23. The entomopathogenic formulation of claim 21 wherein the entomopathogenic fungus comprises a fungus of the class *Hyphomycetes*.

30

24. The entomopathogenic formulation of claim 23 wherein the fungus of the class *Hyphomycetes* is selected from the group of genera consisting of *Beauveria*, *Metarhizium*, *Paecilomyces*,

- 38 -

Culicinomyces, Nomuraea, Sorosporella, Tolypocladium, Verticillium, and Hirsutella.

25. The entomopathogenic formulation of claim 24 wherein
5 the entomopathogenic fungus is selected from the group consisting of
Beauveria bassiana, Metarhizium flavoviride, Metarhizium anisopliae, Paecilomyces fumusoroseus, and Paecilomyces farinosus.

26. The entomopathogenic formulation of claim 21 wherein
10 the emulsion comprises a petroleum based oil and water.

27. The entomopathogenic formulation of claim 26 wherein
the petroleum based oil is a light paraffinic petroleum oil.

15 28. The entomopathogenic formulation of claim 27 wherein
the light paraffinic petroleum oil is Sunspray® 7E Agricultural oil or
Sunspray® 6E Horticultural oil.

29. The entomopathogenic formulation of claim 26 further
20 comprising a clay.

30. The entomopathogenic formulation of claim 29 wherein
the clay is attapulgite clay.

25 31. The entomopathogenic formulation of claim 21 further
characterized by a small volume of the formulation being capable of
spreading over a large area.

30 32. The entomopathogenic formulation of claim 21 further
characterized by a high volume of the formulation being capable of
spreading over a large area.

- 39 -

33. The entomopathogenic formulation of claim 21 wherein the entomopathogenic formulation has a viscosity that allows the entomopathogenic formulation to be sprayed through a nozzle.

5 34. The entomopathogenic formulation of claim 21 wherein the conidia in the formulation retains its entomopathogenic activity for prolonged periods of time.

35. An entomopathogenic formulation comprising conidia of
10 an entomopathogenic fungus, a suspension, and petroleum jelly.

36. The entomopathogenic formulation of claim 35 wherein the emulsifiable suspension is non-phytotoxic at levels used to kill a target insect.

15 37. The entomopathogenic formulation of claim 35 wherein the entomopathogenic fungus comprises a fungus of the class *Hyphomycetes*.

20 38. The entomopathogenic formulation of claim 37 wherein the fungus of the class *Hyphomycetes* is selected from the group of genera consisting of *Beauveria*, *Metarhizium*, *Paecilomyces*, *Culicinomyces*, *Nomuraea*, *Sorospora*, *Tolypocladium*, *Verticillium*, and *Hisutella*.

25 39. The entomopathogenic formulation of claim 38 wherein the entomopathogenic fungus is selected from the group consisting of *Beauveria bassiana*, *Metarhizium flavoviride*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus*, and *Paecilomyces farinosus*.

30 40. The entomopathogenic formulation of claim 35 wherein the suspension comprises a petroleum based oil and water, and clay.

- 40 -

41. The entomopathogenic formulation of claim 40 wherein the petroleum based oil is a light paraffinic petroleum oil.

5 42. The entomopathogenic formulation of claim 41 wherein the light paraffinic petroleum oil is Sunspray® 7E Agricultural oil or Sunspray® 6E Horticultural oil.

43. The entomopathogenic formulation of claim 40 wherein the clay is attapulgate clay.

10

44. The entomopathogenic formulation of claim 35 further characterized by a small volume of the formulation being capable of spreading over a large area.

15 45. The entomopathogenic formulation of claim 35 further characterized by a high volume of the formulation being capable of spreading over a large area.

20 46. The entomopathogenic formulation of claim 35 wherein the entomopathogenic formulation has a viscosity that allows the entomopathogenic formulation to be sprayed through a nozzle.

25 47. The entomopathogenic formulation of claim 35 wherein the conidia in the formulation retains its entomopathogenic activity for prolonged periods of time.

30 48. A method of killing an insect comprising applying to an affected geographical area an entomopathogenic formulation as claimed in claims 1, 13, 21, or 35 such that the population of targeted insect is significantly decreased.

49. The method of killing an insect of claim 48 wherein the entomopathogenic formulation is sprayed through a nozzle.

- 41 -

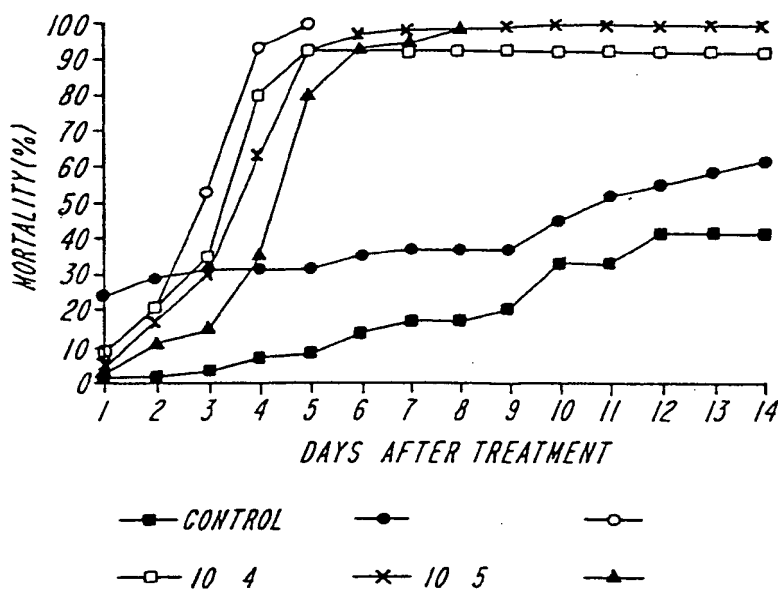
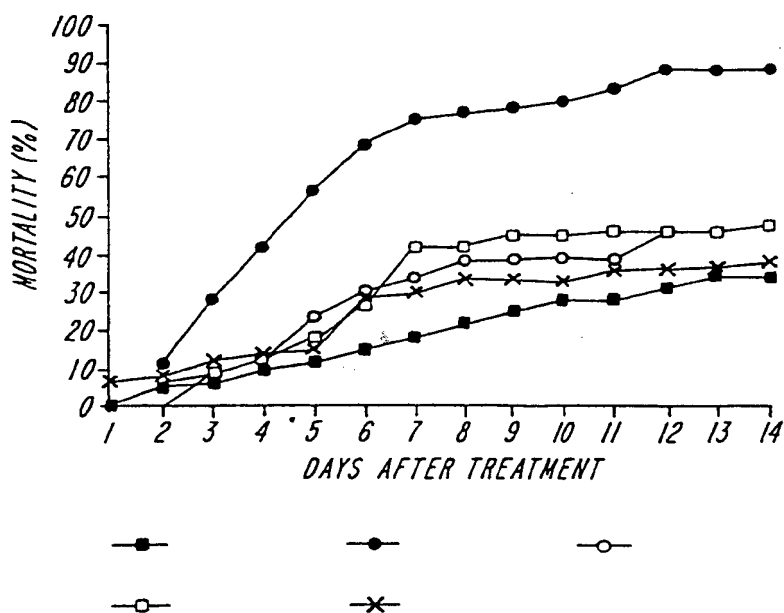
50. The method of killing an insect of claim 48 wherein the insect is an insect selected from the group consisting of grasshopper, locust, white fly, mole cricket, gypsy moth, Colorado potato beetle, corn borer, citrus root weevil, corn root worm, and thrip.

5

51. The method of killing an insect of claim 48 wherein the application takes place by air.

52. The method of killing an insect of claim 48 wherein the
10 application takes place by land.

1/7

**FIG. 1A****FIG. 1B**

2/7

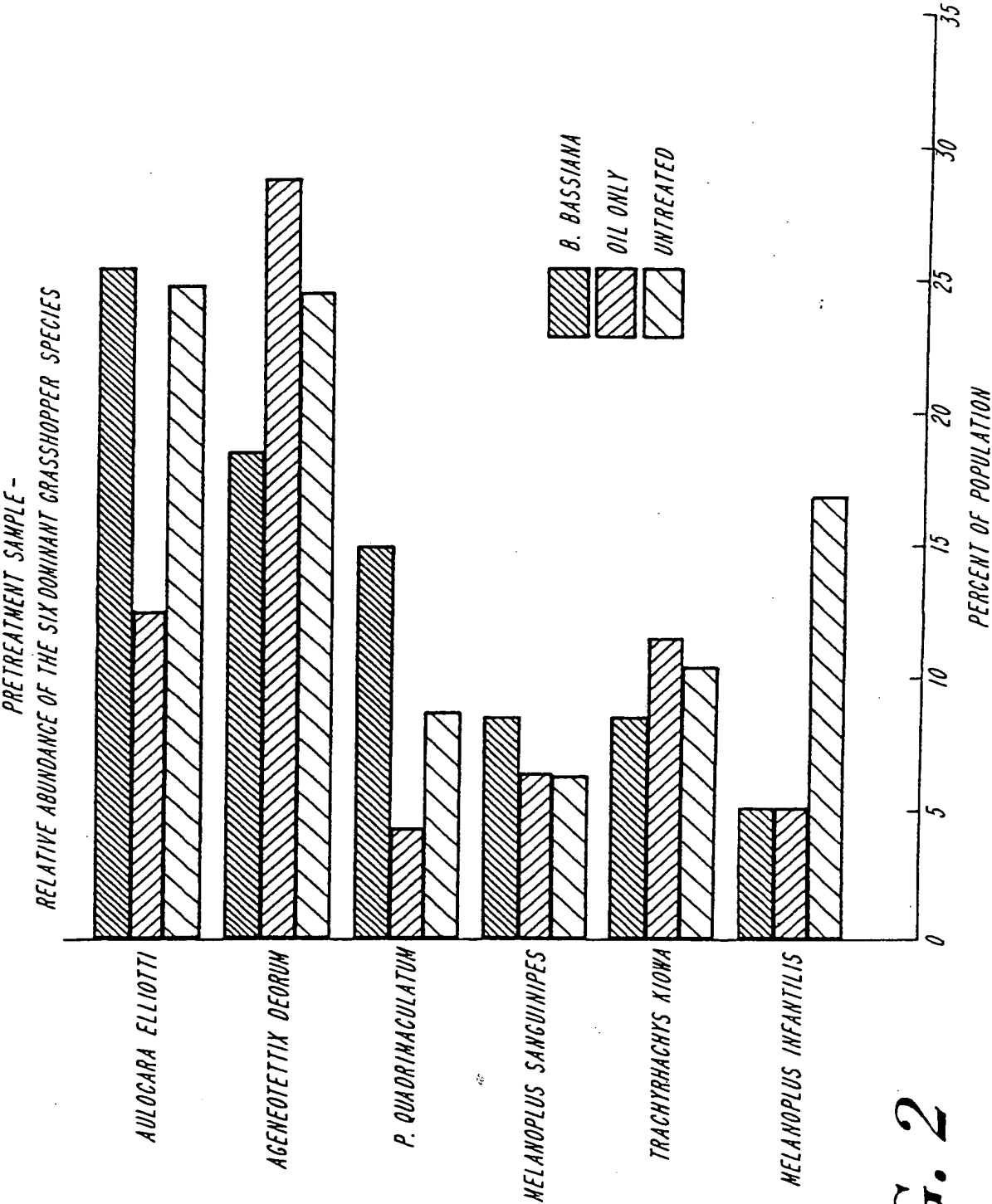


FIG. 2

3/7

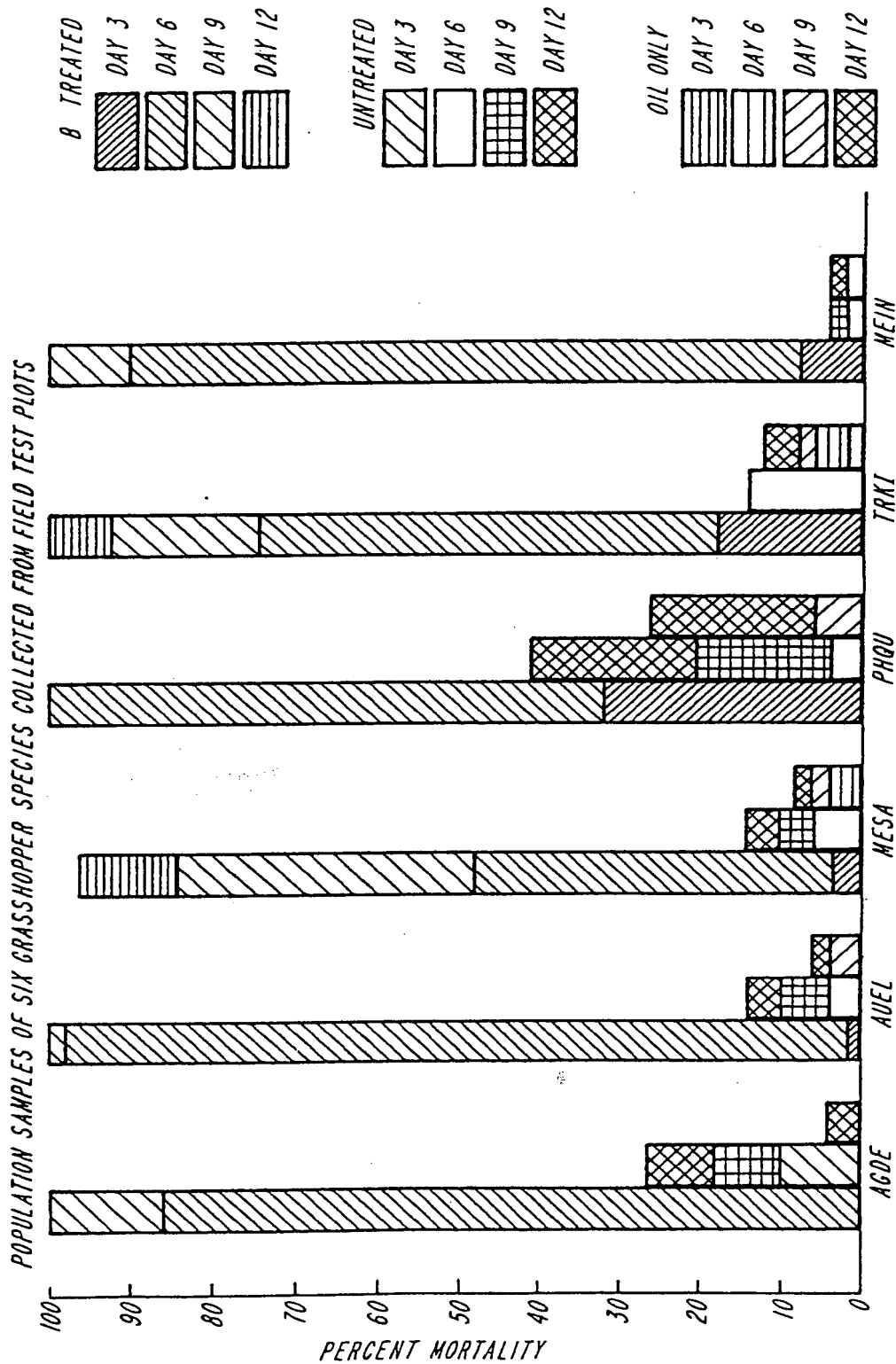


FIG. 3

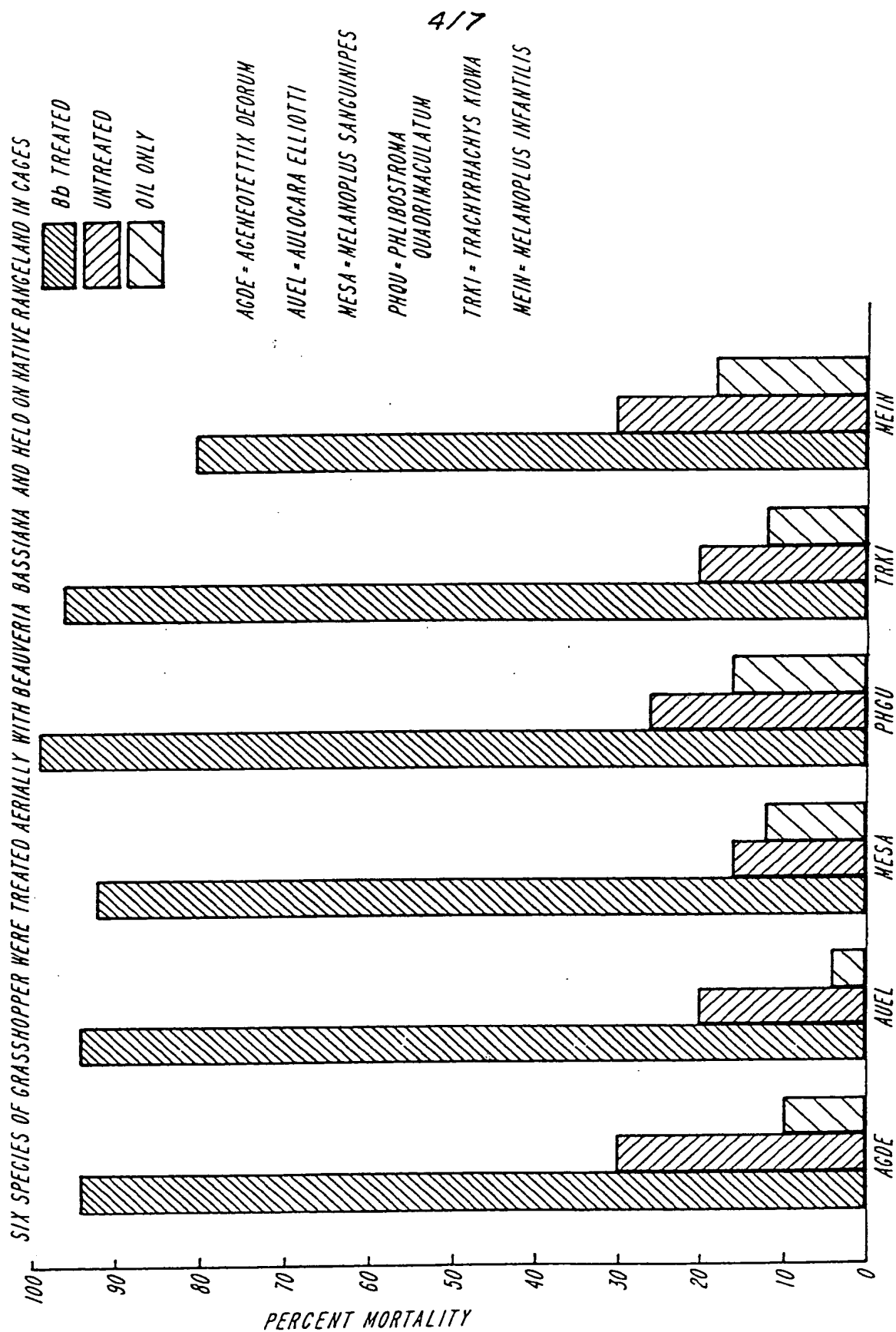


FIG. 4

5/7

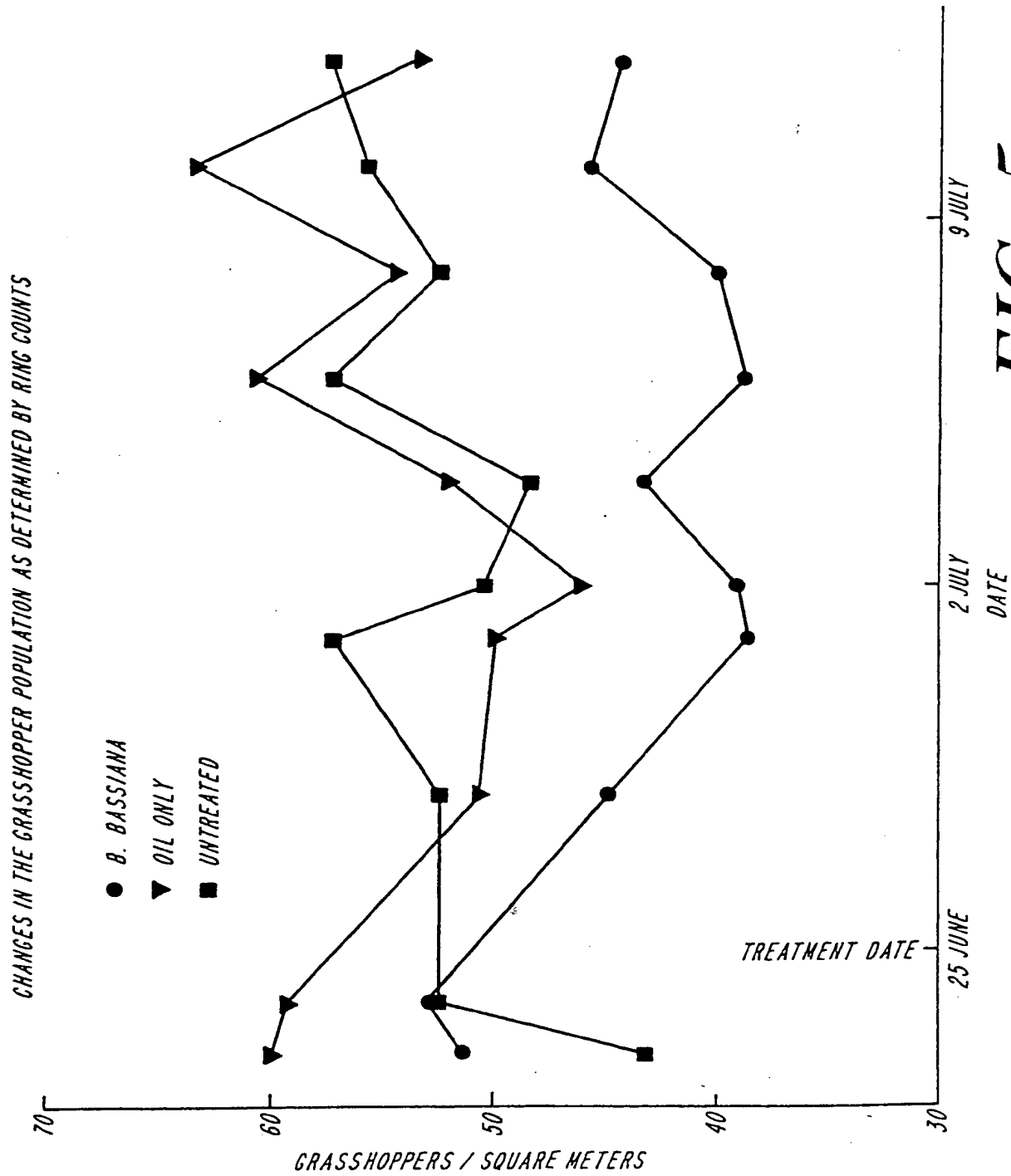
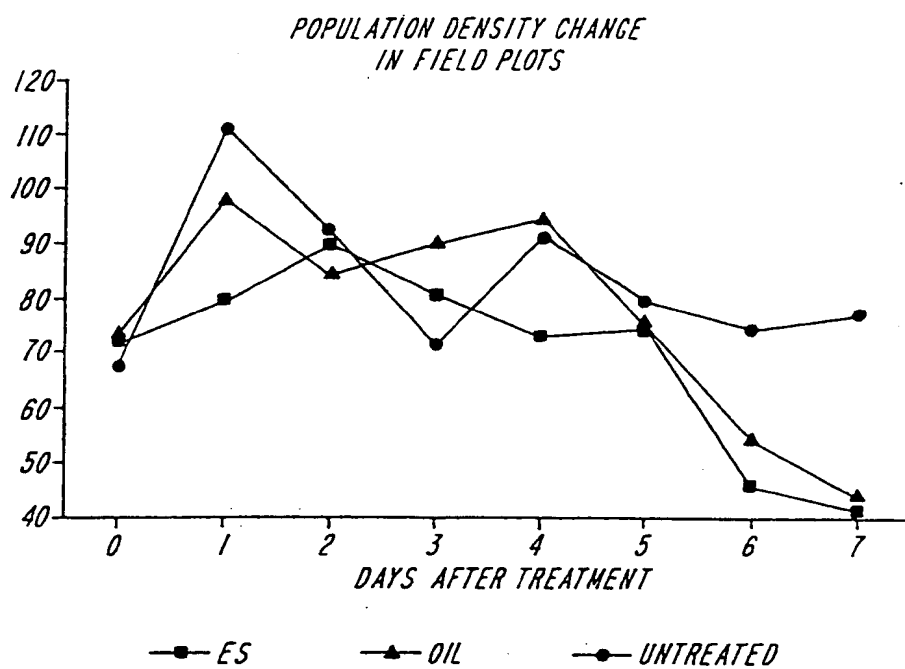


FIG. 5

6/7

**FIG. 6**

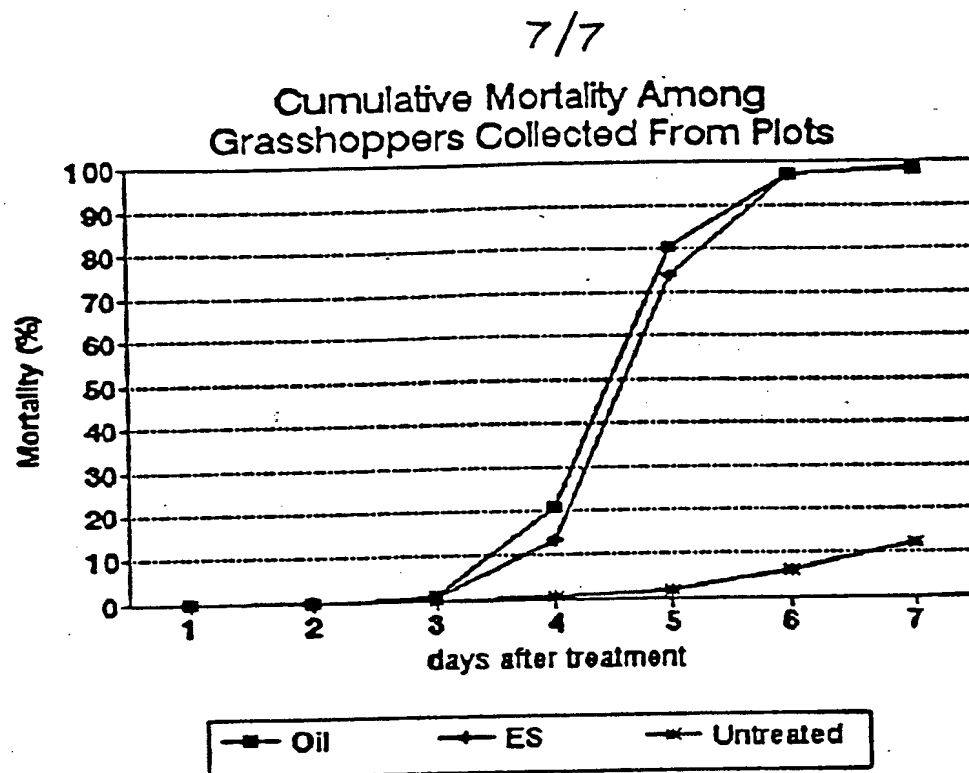


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11542

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 1/14; A01N 63/00, 63/04

US CL : 424/405, 93.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/405, 93.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

search terms: metarhizium or beauveria

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	Weed Technology, Volume 5, issued 1991, W.J. Connick et al., "An Improved Invert Emulsion with High Water Retention for Mycoherbicide Delivery", pages 442-444, see the entire document.	1-6, 8-14, 17-23, 26-28, 31-37, 44-49, 51-52 ----- 7, 15, 16, 24, 25, 29, 30, 38-43, 50
X -- Y	US, A, 4,755,207 (BANNON) 05 July 1988, see the entire document.	1-6, 8-14, 17-23, 26-28, 31-37, 44-49, 51-52 ----- 7, 15, 16, 24, 25, 29, 30, 38-43, 50

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 NOVEMBER 1994

Date of mailing of the international search report

02 FEB 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JEAN C. WITZ

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/11542

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Annals of Applied Biology, Volume 122, issued 1993, R.P. Bateman et al., "The enhanced infectivity of <i>Metarhizium flavoviride</i> in oil formulations to desert locusts at low humidities", pages 145-152, see the entire document.	1-52
Y	US, A, 5,057,316 (GUNNER ET AL.) 15 October 1991, see the entire document.	1-52
Y	Journal of Invertebrate Pathology, Volume 41, issued 1983, R.A. Daoust et al., "Effect of Formulation of the Viability of <i>Metarhizium anisopliae</i> Conidia", pages 151-160, see the entire document.	1-52